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(71) Applicant *(for all designated States except US)*: UNIVERSITY COLLEGE LONDON [GB/GB]; Gower Street, London WC1E 6BT (GB).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: CHARLES, Ian, George [GB/GB]; University College London, Wolfson Institute for Biochemical Research, 140 Tottenham Court Road, London W1P 9LN (GB). BHAGAT, Kiran [GB/GB]; University College London, Wolfson Institute for Biochemical Research, 140 Tottenham Court Road, London W1P 9LN (GB). VALLANCE, Patrick, John, Thompson [GB/GB]; University College London, Wolfson Institute for Biochemical Research, 140 Tottenham Court Road, London W1P 9LN (GB). HINGORANI, Aroon, Dinesh [GB/GB]; University College London, Wolfson Institute for Biochemical Research, 140 Tottenham Court Road, London W1P 9LN (GB).

(74) Agent: WOODS, Geoffrey, Corlett; J.A. Kemp & Co., 14 South Square, Gray's Inn, London WC1R 5LX (GB).

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(54) Title: METHOD FOR IDENTIFYING SELECTIVE INHIBITORS OF NITRIC OXIDE SYNTHASE

(57) Abstract

A method for identifying a compound which selectively inhibits a human nitric oxide synthase (NOS) comprises: (a) determining the inhibition of the activity of the NOS in the presence of a candidate compound and a first concentration of tetrahydrobiopterin (BH_4); (b) determining the inhibition of the activity of the NOS in the presence of the candidate compound and a second concentration of the BH_4; (c) determining whether the candidate compound inhibits the activity of the NOS to a different extent in (a) than in (b).

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METHOD FOR IDENTIFYING SELECTIVE INHIBITORS OF NITRIC OXIDE SYNTHASE

Field of the invention

The present invention relates to methods for identifying compounds which selectively inhibit nitric oxide synthase (NOS). It also relates to the use of such compounds in methods of treatment.

Background to the invention

Septic shock is associated with refractory hypotension and claims over 100,000 lives each year in the USA. Vasodilatation is a ubiquitous and conserved response of the cardiovascular system to acute systemic inflammation, and is the major cause of the hypotension associated with septic shock. Over-production of nitric oxide (NO) has been implicated in the pathophysiology of septic shock in both rodents and man. NO synthesis is catalysed by the enzyme nitric oxide synthase (NOS). There are three known types of mammalian NOS: inducible NOS (iNOS), endothelial NOS (eNOS) and neuronal NOS (nNOS).

In rodent cells, expression of iNOS occurs readily in vascular smooth muscle cells in response to bacterial endotoxin or certain inflammatory cytokines and largely accounts for the hypotension seen in experimental septic shock. Despite the evidence for involvement of iNOS in rodent models, reproducible and consistent induction of functionally active iNOS in human vascular cells and tissues *in vitro* has not been achieved.

Although over-production of NO has been implicated in the pathophysiology of septic shock in man and iNOS is a target for drug therapy, there is no direct evidence that iNOS is expressed in the vasculature of patients with sepsis. Furthermore the plasma concentrations of NOx in patients with sepsis are elevated about 0.5 fold, compared with the 10-fold elevation seen in rodent models. These observations are supported by

molecular evidence for important functional differences between iNOS in different species.

Summary of the invention

The present invention is based on the surprising finding that cytokine induced vasodilatation in humans caused by an increase in NO generation is due to activation of eNOS and not induction of iNOS. This phenomenon appears to be as a result of transcriptional regulation of GTP cyclohydrolase 1 leading to an increase in levels of the pterin tetrahydrobiopterin (BH_4), a co-factor for all three known isoforms of NOS. The increase in levels of BH_4 in turn leads to increased eNOS activity.

The finding that the physiological pathway leading to NO generation by NOSs can be modulated by changes in pterin concentration has specific implications for the design of drugs to treat septic hypotension and inflammatory vasodilatation. It might also be more generally relevant to the route by which excess NO is generated in multiple cell types during systemic inflammation in humans, as well as in the wide range of inflammatory or neoplastic conditions in which pterin synthesis is altered.

Accordingly, the present invention provides a method for identifying a compound which selectively inhibits a nitric oxide synthase (NOS) which method comprises:

(a) determining the inhibition of the activity of the NOS in the presence of a candidate compound and a first concentration of BH_4;

(b) determining the inhibition of the activity of the NOS in the presence of the candidate compound and a second concentration BH_4;

(c) determining whether the candidate compound inhibits the activity of the NOS to a different extent in (a) than in (b).

Preferably step (a) comprises:

(i) determining the activity of said NOS in the absence of the candidate compound and in the presence of a first concentration of BH_4;

(ii) determining the activity of said NOS in the presence of the candidate compound and the first concentration of BH_4;

(iii) comparing the activity of said NOS in steps (i) and (ii).

Preferably step (b) comprises:

(i) determining the activity of said NOS in the absence of the candidate compound and in the presence of a second concentration of BH_4;

(ii) determining the activity of said NOS in the presence of the candidate compound and the second concentration of BH_4;

(iii) comparing the activity of said NOS in steps (i) and (ii).

The second concentration of BH_4 used in step (b) is preferably lower than the first concentration of BH_4 used in step (a). The NOS is preferably a human NOS, more preferably human eNOS.

Compounds identified by the method of the invention which selectively inhibit nitric oxide synthase activity may be used to treat a variety of conditions in which over-production of nitric oxide has been implicated, for example septic shock, asthma, arthritis, inflammatory bowel disease, heart failure and acute systemic inflammation. In addition, this approach might be of value in the treatment of neurological disease states in which nNOS has been implicated (for example, strokes, dementia and Parkinsons disease). Thus, the present invention also provides the use of a compound identified by the method of the invention in the manufacture of a medicament for use in selectively inhibiting a human nitric oxide synthase.

The present invention further provides a pharmaceutical composition comprising a compound identified by the method of the invention together with a pharmaceutically acceptable carrier or diluent.

Cytokine-induced stimulation of NO production by NOS appears to be mediated via an increase in the activity of GTP cyclohydrolase 1 which is the rate-limiting enzyme in the synthesis of BH_4. Since BH_4 is required for NOS activity, and therefore increases in BH_4 levels in response to cytokines result in increased NOS activity, inhibition of GTP cyclohydrolase activity may prevent or reduce NO production by any isoform of NOS.

Thus the present invention further provides the use of a compound in the manufacture of a medicament for use in inhibiting a human nitric oxide synthase wherein said compound is capable of inhibiting or reducing synthesis of BH_4 by GTP

cyclohydrolase 1. Preferably the compound is identified by an assay method which method comprises:

(i) contacting GTP cyclohydrolase 1 with a candidate compound; and

(ii) determining the inhibition of GTP cyclohydrolase-mediated synthesis of BH_4 by said compound.

Detailed description of the invention

The phrase "nitric oxide synthase" (NOS) is intended to include all naturally occuring forms of eNOS, iNOS and nNOS as well as variants which retain NOS activity, for example functional forms of iNOS encoded by additional copies of the iNOS gene or variants produced by mutagenesis techniques. The NOS is preferably of mammalian origin, for example rodent (including rat and mouse) or primate (such as human). Preferably the NOS is of human origin. NOS used in the assays may be obtained from mammal cellular extracts or produced recombinantly from, for example, bacteria, yeast or higher eukaryotic cells including mammalian cell lines and insect cell lines. Preferably, NOS used in the assays is recombinant (see, for example, Charles *et al.*, 1996).

Suitable candidate compounds may include analogues of arginine, aminoguanidine or analogues thereof, guanidine compounds, isothiourea, derivatives of L-N^6-(1-iminoethyl)lysine (L-NMMA), 2-nitroaryl and 2-cyanoaryl compounds or δ-(S-methylisothioureido)-L-norvaline.

Assay methodologies

The inventors have found that the sensitivity of NOS to different inhibitors may vary depending on the physiological pterin concentration. This may be due to an allosteric effect on the conformation of NOS resulting in a BH_4-modified isoform. In particular, as an example, the inventors have found that the rise in physiological BH_4 levels caused by cytokine/endotoxin stimulation increases the sensitivity of eNOS to aminoguanidine. Therefore to provide maximum specificity and clinical efficacy it is very important to screen for potential inhibitors, for use in therapeutic methods, at a physiologically or pathophysiologically relevant pterin concentration.

The principle behind the assay methodologies is to screen for compounds which inhibit a particular NOS isoform at a particular physiologically or pathophysiologically relevant concentration or concentration range of BH_4 but which have a reduced inhibitory effect at other concentrations. By way of an example, the human eNOS isoform which is present at normal BH_4 concentrations is essential for maintaining homeostatic pressure. Any compound which dramatically inhibits this isoform will have a potentially deleterious effect on the patient. However, at increased BH_4 concentrations induced by, for example, inflammatory cytokines, eNOS is converted into a form, possibly due to an allosteric conformational change, which is responsible for generating the NO which causes refractory hypotension. It is this latter form which needs to be targeted. Thus a suitable inhibitor would inhibit eNOS at the pathophysiological concentration of BH_4 present as a result of the action of, for example, inflammatory cytokines but would not inhibit eNOS at normal BH_4 concentrations.

Step (a) of the method of the invention comprises determining the inhibition of NOS activity by the candidate compound in the presence of a first concentration of BH_4. Typically, the amount of NOS used in the assay has a specific activity of from 10 to 600 pmol/min. Typically, the concentration of BH_4 used in the assay is from 1 μM to 1000 μM, preferably from 10 μM to 1000 μM, more preferably from 100 μM to 1000 μM or 500 μM to 1000 μM, most preferably from 500 μM to 1000 μM. Typically, the concentration of candidate compound used in the assay is from 1 μM to 1000 μM, preferably from 1 μM to 100 μM, more preferably from 1 μM to 10 μM.

Generally, inhibition, if any, of NOS activity, is determined by firstly measuring NOS activity in the absence of the candidate compound but in the presence of the first concentration of BH_4. This provides a control value for NOS activity under the assay conditions. Secondly, NOS activity is measured in the presence of both the candidate compound and the first concentration of BH_4. The inhibition of NOS activity due to the candidate compound can be determined by comparing the control value with the value obtained in the presence of the candidate compound. These assay steps are conducted by contacting NOS with the candidate compound and/or BH_4 where appropriate. The extent and/or the rate of reaction can be measured quantitatively, semi-quantitatively or qualitatively. If, for example, a fast throughput screening assay format is required, the

control reaction in the absence of the candidate compound need not be performed for every candidate compound tested.

NOS activity can be measured in a number of ways (see Packer, 1996). For example, NOS activity can be measured directly by conversion of radiolabelled arginine to citrulline or indirectly by assaying for NO production. Assays for NO include chemical assays or bioassays. A typical chemical assay for NO utilises the change in absorbance at 405 nm and 420 nm when NO oxidises oxyhaemoglobin to methemoglobin. Typically, the reaction mixture comprises: 3 μM oxyhaemoglobin, 200 μM $CaCl_2$ (for eNOS and nNOS), 1 mM $MgCl_2$, 1 μM FAD, 1 μM FMN, 100 μM NADPH, 0.1 μM calmodulin, 30 μM L-arginine (substrate for NOS), from 1 μM to 1000 μM BH_4 and 100 μM DTT; in 100 mM HEPES buffer, pH 7.4 and in a final reaction volume of 250 μl (Charles *et al.*, 1996). This assay is suitable for use in a microtitre plate format.

All three isoforms require L-arginine, NADPH, FMN, FAD, BH_4 and calmodulin for activity. eNOS and nNOS also require calcium (Charles *et al.*, 1996).

Step (b) of the method of the invention comprises determining the extent to which the activity of the NOS is inhibited by the candidate compound in the presence of a second concentration of BH_4. This step is similar to step (a) except that the concentration of BH_4 used is different to that used in step (a), preferably lower. Typically, the amount of NOS used in the assay has a specific activity of from 10 to 600 pmol/min. Typically the second concentration of BH_4 used in the assay is from 1 μM to 1000 μM, preferably from 1 μM to 100 μM, more preferably from 1 μM to 10 μM. Typically, the concentration of candidate compound used in the assay is from 1 μM to 1000 μM, preferably from 1 μM to 100 μM, more preferably from 1 μM to 10 μM. Generally the steady-state inhibition is determined after between 15 to 30 minutes incubation.

Step (c) of the method of the invention comprises determining whether the candidate compound inhibits the NOS activity to a different extent in (a) than in (b). This can be accomplished by simply comparing the inhibition in step (a), as indicated by comparing the control value with the second value, and the extent of inhibition in step (b) similarly indicated by comparing the control value with the second value. Thus, for example, a candidate compound may inhibit eNOS by 70% in the presence of a high concentration of BH_4, but only by 5% in the presence of low concentrations of BH_4. Such

a compound would be a useful therapeutic agent because it does not inhibit the normal physiological form of eNOS involved in maintaining homeostatic pressure, but does inhibit the isoform of eNOS which is generated in the presence of high levels of BH_4 caused by inflammatory cytokines/endotoxins.

In other words, NOS activity in the presence of an inhibitor is dependent on (inversely proportional to) the concentration of BH_4. The inhibitor is preferably selective with respect to different isoforms of NOS, for example eNOS, iNOS and nNOS, whether from the same organism or different organisms. For example, an inhibitor may inhibit human eNOS but not human iNOS.

By way of example in numerical form, inhibition of eNOS activity at a higher level of BH_4 by an inhibitor compound is typically at least 20%, preferably at least 30%, more preferably at least 40, 50, 75, 90 or 95% greater than the inhibition of eNOS activity by the inhibitor compound at a lower level of BH_4. The actual concentrations typically envisaged for the higher and lower levels of BH_4 are discussed above.

When screening candidate compounds for use in inhibiting NOS in the treatment of cardiovascular disorders it will be preferable to use eNOS in the assay. Conversely, when screening candidate compounds for use in inhibiting NOS in the treatment of neurological disorders it will be preferable to use nNOS in the assay.

Other types of assay may include *in vivo* assays such as those described in WO95/34534. For example, the inhibition of eNOS and iNOS *in situ* in rat aortic rings can assessed by measuring the increases in ring tension caused by NO synthase inhibition. For studies of basal tone, rings of thoracic aorta with intact endothelium are typically prepared as described in Rees *et al.* (1989) and cumulative concentration curves obtained for the inhibitors in the presence of a threshold concentration of phenylephrine (ED_{10}=10nM). For studies of induced smooth muscle tone, endothelium-denuded rings are typically exposed to LPS (0.1 μg/ml from *S. thphosa*) in the presence of phenylephrine at approximately ED_{90} for 6 h as described in Rees *et al.* (1990). During this time a progressive loss of tone usually occurs because of iNOS induction. Generally, cumulative concentration curves are then obtained for the inhibitors.

The inhibition of eNOS and iNOS *in vivo* can also be assessed by the effects of inhibitors on blood pressure in either normal or endotoxin shocked conscious mice. For

example, one suitable method is as follows. Mice are anaesthetised briefly with isoflourane (2%). Cannula lines are implanted in a femoral vein, tunnelled subcutaneously to exit at the top of the back and connected to a swivel tether system for continuous monitoring of blood pressure and for inhibitor administration respectively. Following recovery from surgery, animals with mean blood pressures in the normal range (90-110 mm Hg) are used to obtain cumulative concentration curves for inhibitors on blood pressure either without further treatment ("normal mice") or 7 h after administration of lipopolysaccharide (12.5 mg/kg of LPS from *E. coli* 026:B6 intravenously over 30 s) to induce shock ("shocked mice"). It will be appreciated that other suitable assays may be used to determine inhibitors of NOS activity *in vivo* (or indeed *in vitro*) such as those described in the examples and that the above mentioned assays are by way of example.

The inventors have also found that cytokine-induced stimulation of NO production by NOS appears to be mediated via an increase in the activity of GTP cyclohydrolase 1 which is the rate-limiting enzyme in the synthesis of BH_4. Since BH_4 is required for NOS activity, and therefore increases in BH_4 levels in response to cytokines result in increased NOS activity, inhibition of GTP cyclohydrolase activity may prevent or reduce NO production by NOS. Thus compounds which inhibit GTP cyclohydrolase 1 activity may also inhibit NOS activity as a result of the inhibition or reduction in BH_4 production.

Generally, inhibition, if any, of GTP cyclohydrolase 1, is determined by firstly measuring GTP cyclohydrolase 1 activity in the absence of the candidate compound. This provides a control value for GTP cyclohydrolase 1 activity under the assay conditions. Secondly, GTP cyclohydrolase 1 activity is measured in the presence of the candidate compound. The inhibition of GTP cyclohydrolase 1 activity due to the candidate compound can be determined by comparing the control value with the value obtained in the presence of the candidate compound. The assays are conducted by contacting GTP cyclohydrolase 1 with the candidate compound. The extent and/or the rate of reaction can be measured quantitatively, semi-quantitatively or qualitatively. If, for example, a fast throughput screening assay format is required, the control reaction in the absence of the candidate compound need not be performed for every candidate compound tested.

Typically, the amount of GTP cyclohydrolase 1 used in the assay is from 1 to 10 ng of pure protein. Typically, the concentration of candidate compound used in the assay

is from 1 μM to 1000 μM, preferably from 1 μM to 500 μM or 1 μM to 100 μM, more preferably from 1 μM to 10 μM.

GTP cyclohydrolase 1 activity can be measured, for example, by assessing the production of BH_4 or GTP consumption. BH_4 production can be determined, for example, by oxidation to biopterin using iodine and measurement of biopterin using HPLC (mobile phase is 5% methanol run at 1.5 ml/min on a 10 micron ODS reverse phase column (4.6 mm x 25 cm) with excitation at 350 nm and emission at 410 nm).

Therapeutic uses

Over-production of nitric oxide as a result of, for example, the effects of bacterial endotoxins, can lead to potentially fatal vasodilatation and hypotension. Selective inhibition of nitric oxide synthases by a compound identified by the method of the invention may be useful in alleviating this potentially fatal response. Compounds identified by the method of the invention may be used therapeutically to treat conditions in which over-production of NO is implicated, for example septic shock, asthma, rheumatoid arthritis, inflammatory bowel disease, heart failure or acute systemic inflammation. NOS is also implicated in various neurological disease states including strokes, dementia and Parkinsons disease.

Similarly, compounds which inhibit or reduce synthesis of BH_4 by GTP cyclohydrolase 1 may also be used therapeutically to treat the conditions described above in which NOS is implicated. Preferably, the compound is used to treat diseases in which eNOS is implicated.

The formulation will depend upon the nature of the compound identified but typically the compounds may be formulated for clinical administration by mixing them with a pharmaceutically acceptable carrier or diluent. For example they can be formulated for topical, parenteral, intravenous, intramuscular, subcutaneous, intraocular or transdermal administration. Preferably, the compound is used in an injectable form. It may therefore be mixed with any vehicle which is pharmaceutically acceptable for an injectable formulation, preferably for a direct injection at the site to be treated. The pharmaceutically carrier or diluent may be, for example, sterile or isotonic solutions. It is also preferred to formulate that compound in an orally active form.

The dose of compound used may be adjusted according to various parameters, especially according to the compound used, the age, weight and condition of the patient to be treated, the mode of administration used and the required clinical regimen. As a guide, the amount of compound administered by injection is suitably from 0.01 mg/kg to 30 mg/kg, preferably from 0.1 mg/kg to 10 mg/kg.

The routes of administration and dosages described are intended only as a guide since a skilled practitioner will be able to determine readily the optimum route of administration and dosage for any particular patient and condition.

The invention will be described with reference to the following examples which are intended to be illustrative only and not limiting. In the accompanying drawings:

Figure 1 - Dose response curves to noradrenaline before and after (a) IL-1β (n=5) (b) IL-1β and TNFα (n=5) or (c) IL-1β, TNFα and IL-6 (n=5)

(a) Dose response curves were constructed to noradrenaline before (o) and 1(□), 6 (□) and 24 h (▲) after instillation of IL-1β (1 ng).

(b) Dose response curves were constructed to noradrenaline before and 1, 6, and 24 h after instillation of IL-1β and TNFα (1 ng) and IL-6 (100 pg).

(c) Dose response curves were constructed to noradrenaline before and 1, 6, 24 and 48h (▼) after instillation of IL-1β (1 ng), TNFα (1 ng) and IL-6 (100 pg).

*indicates $p<0.05$

(d) Dose response curves were constructed to noradrenaline before (o) and 1(□) and 6h (□) after instillation of IL-1β. Six hours after instillation of IL-1β a repeat dose-response curve was constructed with L-NMMA (1 μmol/min; - ▲) co-infused with noradrenaline this was followed by a saline washout period of 15 min and a repeat dose response curve to noradrenaline was constructed with L-arginine (1 μmol/min - ▼) co-infused with noradrenaline.

(e) Dose response curves were constructed to noradrenaline before (o) and 1 (□) and 6h (□) after instillation of IL-1β. Six hours after instillation of IL-1β a repeat dose-response curve was constructed with a aminoguanidine (1 μmol/min - x) co-infused with noradrenaline this was followed by a saline washout period of 15 min and a repeat dose

response curve to noradrenaline was constructed with L-arginine (1 μmol/min - ▾) co-infused with noradrenaline.

Figure 2 - Panel A - Two adjacent vessels were studied simultaneously and at a distension pressure of 40 mmHg. The sympathetic nervous system was activated by asking subjects to take a deep breath. This manoeuvre produced transient constriction in both vessels. Incubation of one vessel (lower trace) with IL-1β virtually abolished sympathetically mediated constriction 6 h later. Sympathetic constriction was restored by infusion of L-NMMA. Congesting cuff deflation and re-inflation is marked by solid circle.

Panel B - Traces showing effects of L-NMMA on basal vessel tone in control vessel (upper trace) and vein treated with IL- 1β (lower trace). The effects of L-NMMA were reversed by L-arginine.

Figure 3 - Panel A - Infusion of BH_4 for 20 min into a control vein causes sustained vasodilatation (hatched line; n=6). Co-infusion of aminoguanidine reverses the dilatation (solid line; n=6).

Panel B - Original trace showing effects of BH_4 infusion (upper trace) and reversal by aminoguanidine (lower trace). Congesting cuff deflation and re-inflation is marked by a solid circle.

Figure 4 - PCR reactions were carried out in 20 μl volumes, and 5 μl samples analysed by electrophoresis through 2% agarose gels.

Panel A shows the result with the iNOS primer pair BB52 and BB71. Track 1 size markers; tracks 2 & 3 mRNA from uninduced hand vein; track 4 and 5 mRNA from cytokine-induced hand vein; track 6 positive control (cytokine-induced CAPAN-1 mRNA). A PCR product of 499 bp is only visible in track 6.

Panel B shows the result of PCR amplification with the eNOS specific primer pair BB158 and BB159. Tracks 1 & 2 mRNA from uninduced vein; tracks 3 & 4 mRNA from cytokine-induced vein; track 5 positive control (human placenta); track 6 size markers. Bands corresponding to eNOS can be detected in tracks 1-5.

Panel C shows the β-actin specific PCR products from the primer set BB27 and BB28. Traces 1-4 are the hand vein mRNA samples amplified with an initial reverse transcriptase step. Tracks 5-8 are negative controls, amplified in the absence of a reverse transcriptase step. Track 9 is a positive control using, placental mRNA. Track 10 is size markers. Bands corresponding to β-actin can be detected in tracks 1-4 confirming that mRNA had been isolated from hand vein biopsies.

EXAMPLE

Materials and methods

In vivo pharmacology

Studies were approved by the local ethics committee and were performed on male and female subjects aged 19-40. Subjects were included who stated that they were healthy and on no medication, and who gave their informed, written consent. Subjects lay supine in a temperature-controlled laboratory (28-30°C). A contesting cuff was placed around the upper arm and inflated to 40 mmHg. Drugs or physiological saline were infused through a 23 gauge needle placed in a dorsal hand vein. The diameter of the vein was measured 5-10 mm downstream from the tip of the infusion needle by recording the linear displacement of a light-weight probe placed on the skin overlying the summit of the vessel when the pressure in the contesting cuff was lowered from 40 to 0 mmHg. In all studies saline was infused for at least 15 min until a stable baseline vein diameter was recorded. To instil cytokines, a length of the vein under study was isolated from the circulation by means of two wedges placed 2-3cm apart on the skin overlying the vessel as described previously, instilled for 1 h, either individually or together. At the end of the period of instillation, the wedges were removed and the vein was reconnected with the circulation for assessment of reactivity. This method of instillation produces local changes in the study vein but adjacent vessels remain unaffected. The volume of blood in the isolated vein is in the order of 1-2 ml and the calculated concentration of cytokine was in the order of 300-1000 pg/ml (TNFα and IL-1β) and 30-100 pg/ml (IL-6).

Biopsy

Surgical removal of the vein was done under local anaesthesia (1% lignocaine™). All samples were handled minimally and immediately frozen to -80°C.

PCR

Reverse transcriptase polymerase chain reaction (RT-PCR) was carried out on poly A^+ mRNA extracted from hand veins using Fast Track reagents (Invitrogen™). Positive control poly A^+ mRNA for eNOS was carried out on human placental preparations (Clontech™). Positive controls for iNOS and GTPCH-1 were carried out on poly A^+ mRNA isolated from the human pancreatic adenocarcinoma line CAPAN-1 stimulated with cytokines. The following primer sets were used:

β actin:

BB27 5'- gattgtgggtatgggtcagaagta-3'

BB28 5'- gctcattgccgatagtgatgacct-3'

GTPCH-1

igc1 5'-ttggttatcttcctaacaag-3'

igc2 5'-gtgctggtcacagttttgct-3'

iNOS

BB52 5' -tccatgcagacaacctt-3'

BB71 5' -gcctcgctctggaaaga-3'

eNOS

BB158 5' -cagtgtccaacatgctgctggaaatt-3'

BB159 5' -taaaggtcttcttcctggtgatgcc-3'

PCR was carried out in thin-walled tubes using a Gene Amp™ RT PCR kit (Perkin-Elmer™) in a Gene Amp PCR system 9600 thermocycler (Perkin-Elmer). The

following conditions were used: 95°C for 1min 45s, followed by 30 cycles of 95°C, 15s, 60°C, 30s. An elongation step of 72°C was carried out for 7 min at the end of the cycle.

Results

Effects of inflammatory cytokines on the vasoconstrictor responses to noradrenaline

We have developed an *in vivo* model to explore mechanisms of septic vasodilatation in man (Bhagat *et al.*, 1996). In this model, drugs or mediators are given into a single superficial vein in very low doses sufficient only to produce changes in the study vessel. This makes it possible to expose a vessel *in situ* to individual cytokines without initiating a systemic cytokine cascade.

First we sought to explore the effects of inflammatory cytokines on the vasoconstrictor responses to noradrenaline. Three cytokines implicated in the systemic cardiovascular response to sepsis were studied. Instillation of IL-1β (1 ng in 1 ml of saline for 1 h) into a single superficial blood vessel caused a rightward shift in the dose response curve to noradrenaline and suppressed the maximal constriction achieved (Figure 1a). This effect developed over several hours and reached a maximum 6 h after exposure of the vessel to IL-1β. By 24 h the potency of noradrenaline was fully restored. Instillation of either tumour necrosis factor α (TNFα); 1 ng in 1 ml of saline), or IL-6 (100 pg, in 1 ml of saline) alone produced no significant change in the response to noradrenaline (data not shown), but co-instillation of these cytokines with IL-1β (1 ng/ml) for 1 h caused a prolonged (>24 h) attenuation of the response to noradrenaline (Figure 1b). The slowly developing hyporesponsiveness to noradrenaline was prevented by prior treatment of subjects with oral hydrocortisone (100 mg, given 2 h before instillation of cytokine; n=6, data not shown). These studies identify IL-1β as a key cytokine inducing vascular changes in man and indicate that a therapeutically relevant dose of a glucocorticoid prevents the development of the vasorelaxation.

Effect of NOS inhibitors on IL-1β-induced hyporesponsiveness to noradrenaline.

To determine whether increased generation of NO contributed to the hyporesponsiveness to noradrenaline, we tested the effects of N^G monomethyl-L-arginine (L-NMMA; a standard NOS inhibitor) , and aminoguanidine (a compound that has been widely used as a selective inhibitor of iNOS). Both L-NMMA (1 μmol/min) and aminoguanidine (1 pmol/min) reversed the IL-1β-induced attenuation in the dose response curve to noradrenaline (Figure 1). Infusion of the substrate for NOS (L-arginine; 1 μmol/min) reversed the effect of L-NMMA or aminoguanidine (Figure 1). In control veins (not treated with IL-1β) L-NMMA (1 μmol/min) and aminoguanidine (1 μmol/min) were devoid of constrictor action (Figure 2B) and did not alter the response to noradrenaline (data not shown), confirming previous observations that these vessels do not generate NO basally. As expected, in control veins not exposed to cytokines, L-NMMA (1 μmol/min) inhibited the dilatation produced by the endothelium-dependent dilator bradykinin by 88%, whereas aminoguanidine (1 μmol/min) did not. Together these studies demonstrate that the hyporesponsiveness induced by IL-1β is due to generation of NO. The time course and pharmacological profile of the response (prevention by glucocorticoids, reversal by L-NMMA and aminoguanidine) are consistent with expression of iNOS, and have been taken as indicative of such in many previous studies.

The potential physiological significance of the increase in NO synthesis induced by IL-1β is illustrated in Figure 2A. Instillation of cytokines virtually abolished endogenous venoconstriction due to transient activation of the sympathetic nervous system (achieved by asking subjects to take and hold a deep breath), sympathetic nervous system activity is a major determinant of venous tone in humans and sympathetic blockers cause profound venodilatation and postural hypotension. Our findings indicate that the profound venodilatation that accompanies sepsis or a systemic inflammatory response is likely to be mediated by an induction of basal generation of NO within the vessel wall that blunts the ability of the sympathetic nervous system to alter venous tone. Local infusion of

L-NMMA did not alter control responses to sympathetic activation, but restored the ability of the sympathetic nervous system to cause venoconstriction in vessels exposed to IL-1β (Figure 2A). This explains the observation that systemic administration of L-NMMA does not alter venous pressure in healthy volunteers but increases it in patients with septic shock.

eNOS is sensitive to inhibition by an iNOS inhibitor, aminoguanidine, in the presence of BH_4.

In a separate series of studies we found that infusion of BH_4 (a co-factor for all 3 isoforms of NOS) into preconstricted healthy control veins caused rapid vasodilatation (Figure 3). In contrast to bradykinin-induced vasodilatation, this dilator response to BH_4 was fully reversed by infusion of aminoguanidine (1 μmol/min; Figure 3). Since control vessels would be expected to express eNOS, but not iNOS, and the vasodilatation produced by BH_4 was rapid and consistent with activation of a constitutive enzyme, this finding apparently presents a conundrum. However, whilst aminoguanidine had little effect on the activity of purified human eNOS in the absence of BH_4, as BH_4 was added to the enzyme, aminoguanidine became an effective inhibitor (data not shown). Furthermore, addition of BH_4 to human endothelial cells in culture increased generation of NO and this was inhibited by aminoguanidine. Thus, the ability of aminoguanidine to inhibit eNOS activity is dependent on the prevailing concentration of BH_4.

IL-1β induces expression of GTP cyclohydrolase 1 leading to increased generation of BH_4.

The rate limiting enzyme for BH_4 synthesis is GTP cyclohydrolase 1. This enzyme is transcriptionally regulated in response to cytokines or endotoxin, and its induction can be prevented by glucocorticoids. It is clear, therefore that the pharmacological changes we observed in the hand vein *in vivo* might be explained equally well by activation of eNOS by BH_4 synthesised following induction of GTP cyclohydrolase 1, as by induction of iNOS. To explore further the mechanism of the functional induction of NOS activity we excised a portion of hand vein. As expected, mRNA encoding eNOS was detected in all of the samples (Figure 4). Messenger RNA encoding GTP cyclohydrolase 1 was detected

in veins exposed to cytokines (Figure 4). In contrast, under identical conditions, no mRNA encoding iNOS was detected (Figure 4). Taken together, the simplest explanation of the pharmacological and molecular studies is that IL-1β induces expression of GTP cyclohydrolase 1 which leads to increased generation of BH_4 and consequent activation of eNOS. This interpretation is further strengthened by the finding that treatment of human cultured endothelial cells with IL-1β leads to appearance of mRNA for GTPCH-1 but not iNOS and to induction of NO generation as assessed by measurement of nitrite in the culture medium. FACS analysis showed that the cells expressed eNOS protein but iNOS protein was not detected. It is not clear whether the mRNA for GTP cyclohydrolase 1 we detected in the hand veins was present in the vascular endothelium, the smooth muscle, or throughout the vessel wall, but it has been shown that BH_4 generated in one cell can enter neighbouring cells to activate NOS. Neopterin levels are greatly increased in the plasma of patients with infections and activated human macrophages make significant amounts of neopterin and biopterin.

Our study clearly identifies IL-1β as a key cytokine causing physiologically significant vasodilatation in humans by increasing NO Generation. We suggest that the phenomenon is explained by transcriptional regulation of GTP cyclohydrolase 1 and the consequent activation of eNOS by BH_4. eNOS is an endothelial enzyme that has not been detected in smooth muscle cells. An implication of our findings is that the endothelium can generate sufficient NO to cause profound vasodilatation and that expression of NOS throughout the much larger smooth muscle cell layer is not necessary to cause septic hypotension.

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CLAIMS

1. A method for identifying a compound which selectively inhibits a nitric oxide synthase (NOS) which method comprises:

(a) determining the inhibition of the activity of the NOS in the presence of a candidate compound and a first concentration of tetrahydrobiopterin (BH_4);

(b) determining the inhibition of the activity of the NOS in the presence of the candidate compound and a second concentration of the BH_4;

(c) determining whether the candidate compound inhibits the activity of the NOS to a different extent in (a) than in (b).

2. A method according to claim 1 wherein step (a) comprises

(i) determining the activity of said NOS in the absence of the candidate compound and in the presence of a first concentration of BH_4;

(ii) determining the activity of said NOS in the presence of the candidate compound and the first concentration of BH_4; and

(iii) comparing the activity of said NOS in steps (i) and (ii).

3. A method according to claim 1 or 2 wherein step (b) comprises

(i) determining the activity of said NOS in the absence of the candidate compound and in the presence of a second concentration of BH_4;

(ii) determining the activity of said NOS in the presence of the candidate compound and the second concentration of BH_4;

(iii) comparing the activity of said NOS in steps (i) and (ii).

4. A method according to claim 1, 2 or 3 wherein said second concentration is lower than said first concentration.

5. A method according to any one of the preceding claims wherein said NOS is human eNOS.

6. A method according to any one of claims 1 to 5 wherein said NOS is human iNOS.

7. A method according to any one of claims 1 to 5 wherein said NOS is human nNOS.

8. Use of a compound identified by the method of any one of the preceding claims in the manufacture of a medicament for use in selectively inhibiting a human nitric oxide synthase.

9. Use according to claim 8 wherein said NOS is eNOS.

10. Use according to claim 8 wherein said NOS is iNOS.

11. Use according to claim 8 wherein said NOS is nNOS.

12. Use of a compound identified by the method of any one of claims 1 to 7 in the manufacture of a medicament for use in treating toxic shock syndrome.

13. A method for identifying a compound which is an inhibitor of GTP cyclohydrolase 1- mediated synthesis of BH_4 which method comprises:

(i) contacting GTP cyclohydrolase 1 with a candidate compound; and

(ii) determining the inhibition of GTP cyclohydrolase 1-mediated synthesis of BH_4 by said compound.

14. Use of a compound in the manufacture of a medicament for use in inhibiting a human nitric oxide synthase wherein said compound is capable of inhibiting or reducing synthesis of BH_4 by GTP cyclohydrolase 1.

15. Use according to claim 14 wherein said compound is identified by an assay method which method comprises:

(i) contacting GTP cyclohydrolase 1 with a candidate compound; and

(ii) determining the inhibition of GTP cyclohydrolase 1-mediated synthesis of BH_4 by said compound.

16. Use according to claim 14 or 15 wherein said NOS is eNOS.

17. A compound identified by the method of any one of claims 1 to 7 or 13.

18. A compound according to claim 17 for use in therapy.

19. A compound according to claim 18 for use in treating toxic shock syndrome.

20. A compound according to claim 17 for use in the manufacture of a medicament for prophylaxis or treatment of a clinical condition caused by excessive NO production.

21. A pharmaceutical composition comprising a compound according to any one of claims 17 to 20 together with a pharmaceutically acceptable carrier or diluent.

22. A method for preventing or treating a clinical condition in a patient caused by excessive NO production which method comprises administering an effective amount of a compound according to any one of claims 17 to 20 or a pharmaceutical composition according to claim 21, to said patient.

Fig.1.

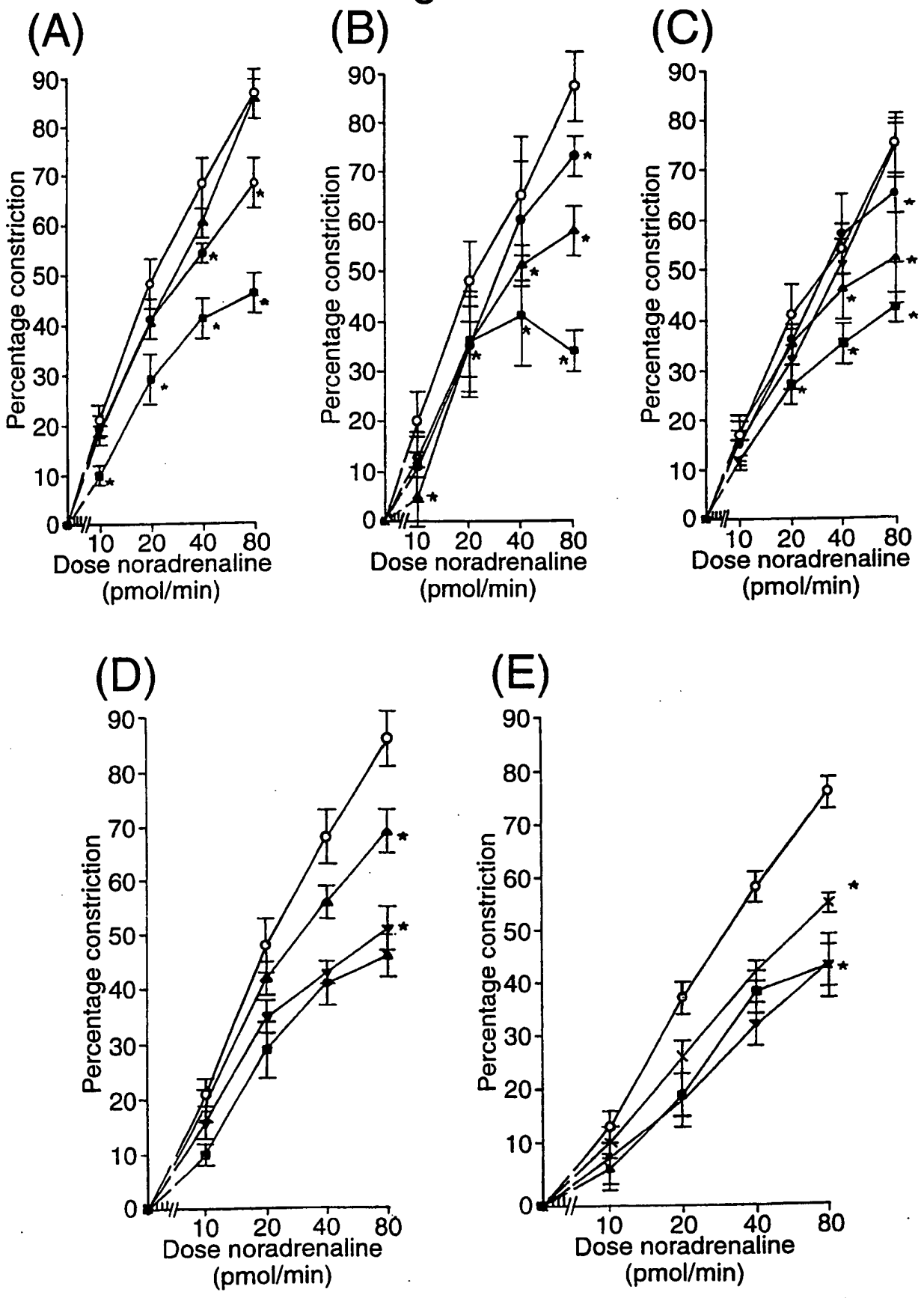

(A)
(B)
(C)
(D)
(E)
Percentage constriction
0
10
20
30
40
50
60
70
80
90
10 20 40 80
Dose noradrenaline
(pmol/min)

Fig.2.

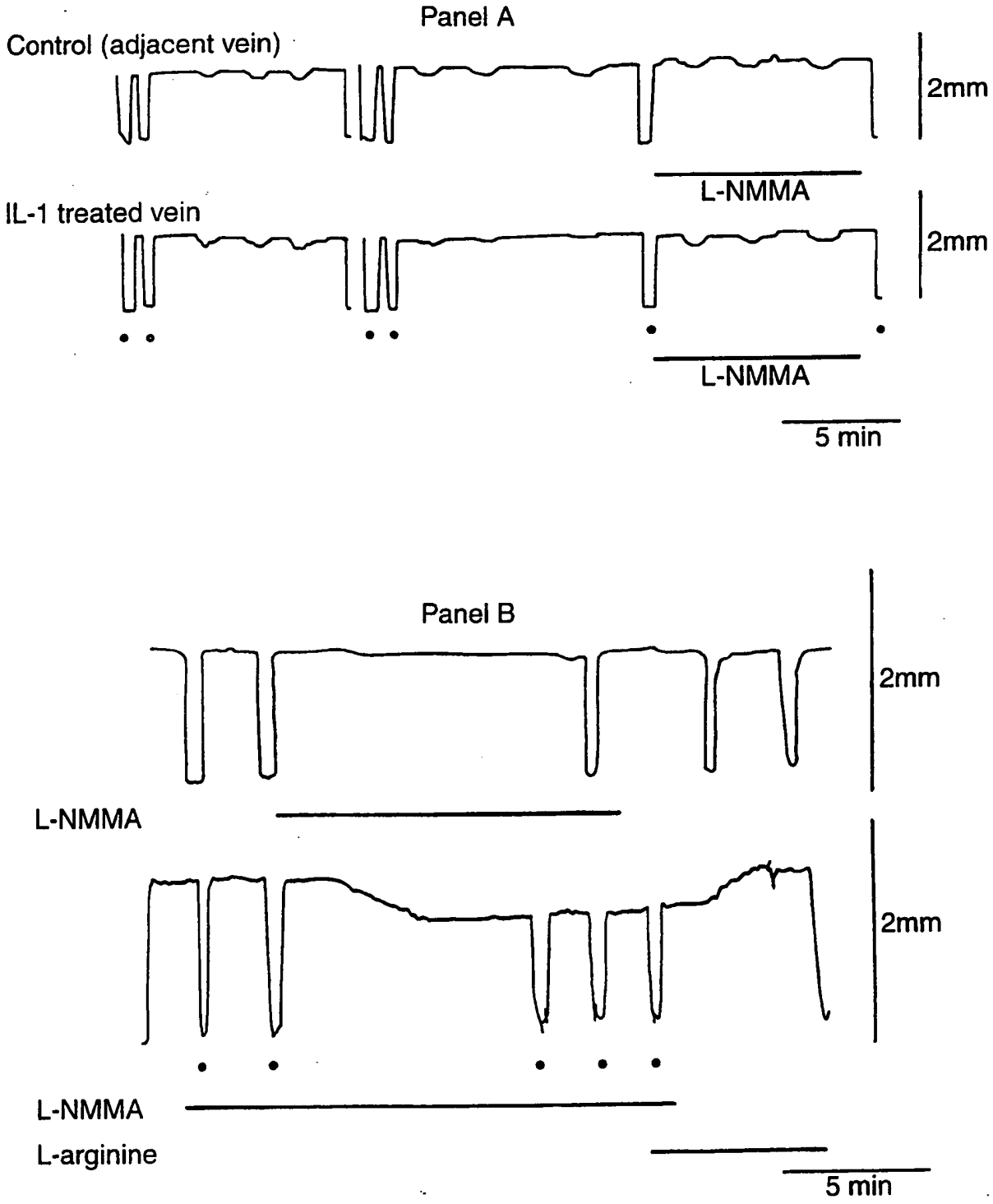
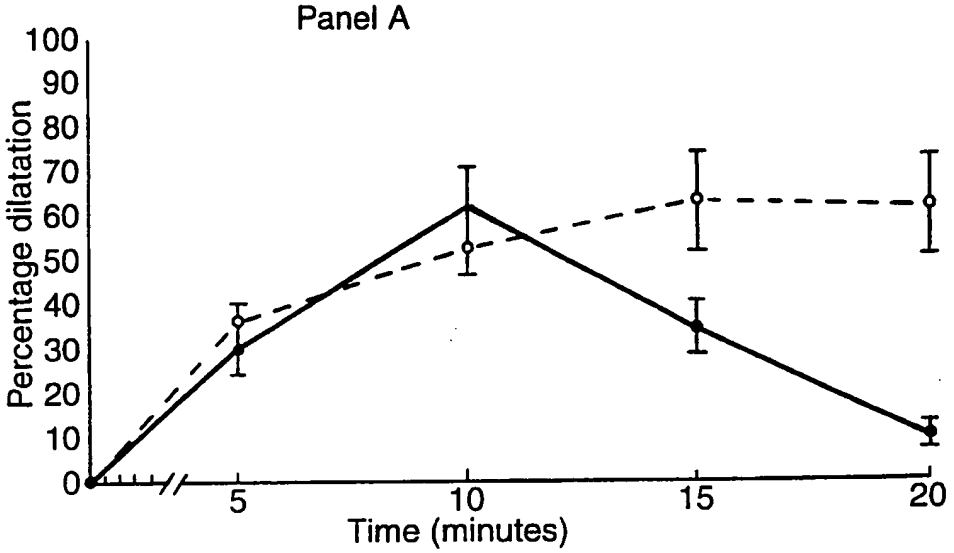

Panel A
Control (adjacent vein)
2mm
L-NMMA
IL-1 treated vein
2mm
L-NMMA
5 min
Panel B
2mm
L-NMMA
2mm
L-NMMA
L-arginine
5 min

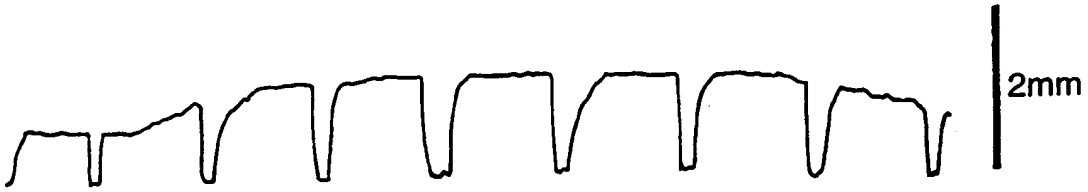
Fig.3.

Panel A

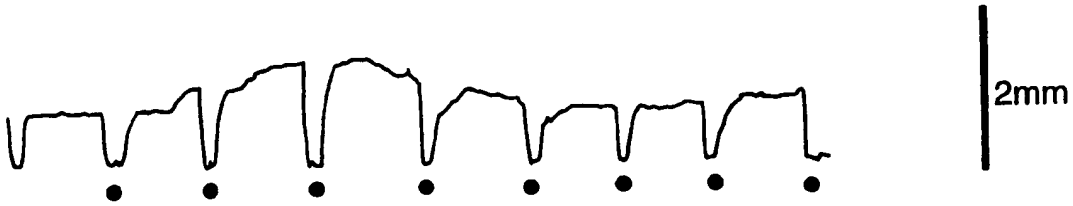


BH4 (250nmol/min)

Aminoguanidine 1pmol/min



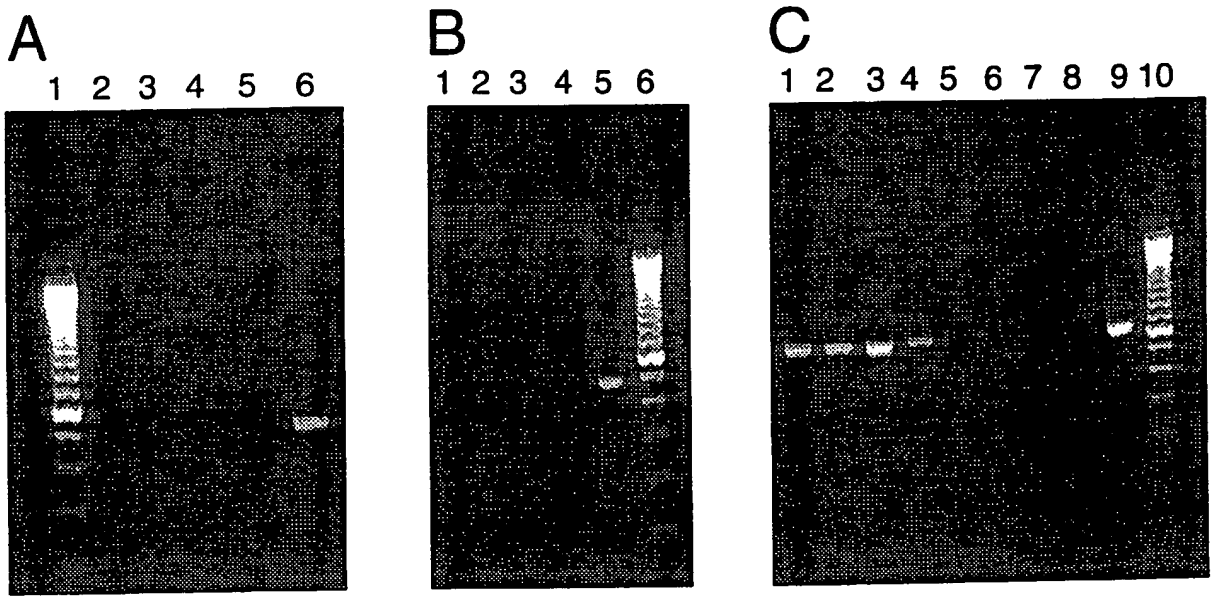
BH4



BH4

Aminoguanidine

Fig.4.



INTERNATIONAL SEARCH REPORT

Int tional Application No
PCT/GB 98/00353

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/26 C12Q1/34 A61K31/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	P. KLATT, M. SCHMID, E. LEOPOLD, K. SCHMIDT, E. R. WERNER, B. MAYER: "The Pteridine Binding Site of Brain Nitric Oxide Synthase" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 269, no. 19, 13 May 1994, pages 13861-13866, XP002066035 see page 13863 see page 13865 --- -/--	1-12, 17-22

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
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Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl. Fax: (+31-70) 340-3016	Hart-Davis, J

2

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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INTERNATIONAL SEARCH REPORT

Information on patent family members

Int ional Application No
PCT/GB 98/00353

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